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(54) Title: METHOD FOR GENETIC MANIPULATION

(57) Abstract: Capped RNA corresponding to the spliced transcript of the *Minos* transposable element has been synthesised *in vitro* and shown to be active as a source of transposase for *Minos* transposon mobilisation. When co-injected into *Drosophila* and Medfly embryos, *Minos* mRNA can mobilise homologous transposons from plasmids into germline chromosomes. Injected mRNA is superior to injected plasmids expressing the transposase as a source of the enzyme, resulting in several-fold increases in transformation efficiencies. In a rapid test for mRNA activity, *Minos* mRNA has been shown to catalyse excision of a *Minos* transposon from plasmid DNA in Medfly embryos. High frequency mobilisation of a *Minos* transposon from the X chromosome into autosomes has also been demonstrated after injections of *Minos* transposase mRNA into pre-blastoderm *Drosophila* embryos. The rates of transposition (approximately 30 %) suggest that this is a powerful system for genetic manipulation.

WO 01/29205 A2



METHOD FOR GENETIC MANIPULATION

The present invention relates to a method for the transfer of genetic information in an organism using transposons. In particular, the method according to the invention
5 comprises the provision of a transposase activity, to catalyse transposon mobilisation, in the form of a ribonucleic acid encoding the transposase.

The introduction of exogenous DNA into the genome is a critical step for the study of molecular genetics of multiple insect species. Until recently, the absence of convenient
10 and effective methodology applied to insects other than *Drosophila* has been a serious drawback in such studies. In *Drosophila melanogaster* and closely related species, the P-element has been used widely to mediate germ line transformation (1). Despite its efficiency in *Drosophila*, the P transformation system does not function in non-*Drosophila* species. Transgenic technology based on mobile elements other than P is currently
15 applied to diverse insect species of agricultural or medical importance. Over the past few years several Type II transposable elements have been used for germline transformation in species other than their natural hosts, such as *Minos* (see below), *mariner* in *D. melanogaster*, *D. virilis* and *Aedes aegypti* (2-4); *Hermes* in *D. melanogaster* and *Ae. aegypti* (5-6) *Hobo* in *D. virilis* (7); *piggyBac* in *D. melanogaster*, *C. capitata* and *Bombyx mori* (8-10). Moreover, purified transposase from the *Tc1*, *Mos1*, and *Himar1*, members
20 of the *Tc1*/*mariner* superfamily, can catalyse transposition of the homologous transposons *in vitro* (11-13). These results indicate that apart from the transposase, the transposition of these elements may not require other species-specific factors employed by the host cells. It is generally believed that these elements do not face severe host
25 restrictions.

Minos, a member of the *Tc1* family of elements, was isolated from *D. hydei* and is absent from both *D. melanogaster* (14) and *C. capitata* (Unpublished data). *Minos* has been used for the germ line transformation of *D. melanogaster* and *C. capitata* (15-16) and
30 using transient mobilisation assays it has also been shown to be active in embryos of *D. melanogaster*, *Aedes aegypti*, *Anopheles stephensi* and *Bombyx mori* and in cell lines of *D. melanogaster*, *Aedes aegypti*, *Anopheles gambiae* and *Spodoptera frugiperda* (17-19).

35 The standard methodology for transposable element mediated transformation is by co-injecting into pre-blastoderm embryos a mixture of two plasmids: one expressing

transposase (Helper) but unable to transpose, and one carrying the gene of interest flanked by the inverted terminal repeats of the element (Donor). Transformed progeny of injected animals is detected by the expression of dominant marker genes. Such methods, however, provide very low efficiencies of transformation.

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For species in which DNA introduction into embryos is easy, low transformation frequencies would not be a major practical problem. Nevertheless, in those cases that the introduction is not trivial, either due to small numbers of available eggs or to other factors, high transformation frequencies may be critical for success.

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Tc1/*mariner* elements have a number of advantages over viral and non-viral gene delivery systems, such as the stable, single-copy integration of transgenes into chromosomes, less strict maximal insert sizes, precise regulation of transposition by controlling the expression of transposase, increased accessibility of diverse tissue cells (20). Re-mobilisation of transgenes by the introduction of functional transposase (21-22) and germ line transformation with transposon vectors support powerful methodologies in molecular genetic research, such as analysis of genes either through loss-of-function (23-24) or gain-of-function (25-26) insertional mutagenesis, gene cloning by transposon tagging (27) and enhancer trapping (28-29). Random integration of transgenes into chromosomes is a clear advantage for these applications, if the frequency of transposition is not a limiting factor.

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There remains a need, however, for a transposon-mediated transformation system in which the efficiency of transformation is increased. In WO 99/07871 a transformation system based on Tc1/*mariner* elements has been proposed. Although the authors suggest that a transposase activity may be provided on a DNA vector, as mentioned above, transposon mobilisation by this technique is not demonstrated. Instead, transposase protein is produced in foreign hosts, purified and administered directly to cells harbouring the transposons. Such a technique has evident disadvantages, since transposase is not easily isolatable from natural sources, and recombinantly-derived transposase is shown to give a low efficiency of transformation.

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Moreover, there remains a need for a method which can efficiently remobilise resident transposons in a cell. The methods described in WO 99/07871 are not demonstrated to be able to successfully remobilise integrated transposons in a cell.

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Summary of the Invention

We have now developed a new transposon-mediated transformation method which provides highly efficient mobilisation of transposons of the *Tc1/mariner* class, in particular *Minos*. The invention provides the transposase activity in the form of RNA
5 encoding the transposase, coadministered to the cell with the transposon.

In a preferred embodiment of the invention, *in vitro* synthesised *Minos* mRNA is used as a source of *Minos* transposase, which offers the advantage of transient, increased levels
10 of transposase, and eliminates the danger of random integration of the helper plasmid into the genome through non-homologous recombination. The transposase encoded by *Minos* mRNA can mobilise *Minos* transposons from plasmids co-injected in *Drosophila* embryos. Moreover, high frequencies of germ line transformation and re-mobilisation of a resident *Minos* transgene are achieved by this method.

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According to a first aspect of the invention, therefore, there is provided a method for genetically modifying a cell by transposon mobilisation, comprising the steps of:

- a) delivering to the cell a nucleic acid comprising a transposon of the *Tc1/mariner* class;
- 20 b) delivering to the cell a ribonucleic acid encoding a cognate transposase for said transposon; and
- c) causing the ribonucleic acid to be translated to produce the cognate transposase within the cell.

25 According to a second aspect of the invention, there is provided a method for mobilising a transposon resident in a host cell genome, comprising the steps of:

- a) delivering to the cell a ribonucleic acid encoding a cognate transposase for said transposon; and
- b) causing the ribonucleic acid to be translated to produce the cognate
30 transposase within the cell, thus mobilising the resident transposon.

In the context of the present invention, delivery of the nucleic acids may be accomplished by any available technique, including transformation/transfection, delivery by viral or non-viral vectors and microinjection. Each of these techniques is known in the
35 art. Ribonucleic acids, in particular, may be delivered by microinjection or by viral

transduction, particularly by RNA viruses in which the viral genome comprises the ribonucleic acid encoding the transposase.

5 A nucleic acid, as referred to herein, may be any nucleic acid, including DNA and RNA, as well as synthetic nucleic acid homologues such as backbone-modified nucleic acids including methylphosphonates, phosphorothioates and phosphorodithioates, where both of the non-bridging oxygens are substituted with sulphur; phosphoroamidites; alkyl phosphotriesters and boranophosphates. Achiral phosphate derivatives include 3'-O'-5'-S-phosphorothioate, 3'-S-5'-O-phosphorothioate, 3'-CH₂-5'-O-phosphonate and 10 3'-NH-5'-O-phosphoroamidate. Peptide nucleic acids replace the entire phosphodiester backbone with a peptide linkage.

Sugar modifications are also used to enhance stability and affinity. The α -anomer of deoxyribose may be used, where the base is inverted with respect to the natural 15 β -anomer. The 2'-OH of the ribose sugar may be altered to form 2'-O-methyl or 2'-O-allyl sugars, which provides resistance to degradation without comprising affinity.

Modification of the heterocyclic bases must maintain proper base pairing. Some useful substitutions include deoxyuridine for deoxythymidine; 5-methyl-2'-deoxycytidine and 20 5-bromo-2'-deoxycytidine for deoxycytidine. 5-propynyl-2'-deoxyuridine and 5-propynyl-2'-deoxycytidine have been shown to increase affinity and biological activity when substituted for deoxythymidine and deoxycytidine, respectively.

A ribonucleic acid, as referred to herein, may be natural or modified RNA. 25 Advantageously, the RNA may comprise one or more of the modifications identified above.

Translation of the ribonucleic acid to produce the transposase is preferably effected by endogenous cellular translation machinery. This may be under the control of natural 30 cellular factors and sequences, and/or, particularly where the RNA is delivered by viral transduction, of viral sequences and factors.

The cell may be any suitable cell type, including plant, insect and mammalian cells. The cells may be part of an organism, in primary culture, or established cell lines. Insect cells 35 are preferred. The method of the present invention may be used to create transgenic organisms, such as transgenic insects, mammals or plants, by delivering to the oocyte a

nucleic acid comprising a transposon and a ribonucleic acid encoding the transposase activity.

Preferably, the transposon is selected from the group consisting of *Minos*, *mariner*,
5 *Hermes* and *piggyBac*. Advantageously, the transposon is *Minos*. Each transposon is advantageously employed with its natural cognate transposase, although the use of modified and/or improved transposases is envisaged.

The transposon preferably comprises a nucleic acid sequence encoding a heterologous
10 polypeptide. This sequence will be integrated, together with the transposon, into the genome of the cell on transposon integration. Moreover, it will be excised, together with the transposon, when the latter excises on remobilisation. In a preferred embodiment, the heterologous polypeptide is a selectable marker. This allows cells having integrated transposons to be identified and the site of integration to be accurately mapped.

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Brief Description of the Figures

Figure 1. A) Structure of the C58 insertion. B) Mating schemes for the detection of
excision and transposition of the stable *Minos* insertion (black arrowhead) in the C58
20 strain. For more information see Materials & Methods.

Figure 2. *Minos*-transposon excision from a donor plasmid mediated by *Minos*-
transposase mRNA. Excision scheme and PCR results from medfly embryos. Lane 1;
No DNA (negative control of PCR), Lane 2; Non-injected embryos, Lane 3; injected with
25 *Minos*-mRNA/donor mix embryos (pool No I), Lane 4; injected with *Minos*-mRNA/donor
mix embryos (pool No II), Lane 5; Excision in S2 cells (positive control of the PCR), Lane
6; injected with donor plasmid (pool V), Lane 7; injected with donor plasmid (pool VI).
Excision results in a PCR product of 211 bp while the 2.2 Kb band results from the
amplification of an intact transposon sequence. The band at ~1.2 Kb is the result from
30 ectopic priming from the donor plasmid sequences.

Figure 3. (A) *MihsCcw* transposon. Medfly *white* cDNA and *Hsp70* (promoter and
terminator) sequences, are shown in white. Black arrows indicate the *EcoRI* restriction
sites. Black bars above the map indicate the *Minos* sequences that were used as probe
35 for the analysis of transformants. (B) Southern blot analysis of *Ceratitis capitata*
transformants. DNA from G2 transformed flies and the *w* recipient strain was digested

with *EcoR* I and hybridized with *Minos* probe (see Materials & Methods). Brackets indicate G1 flies derived from the same G0 cage. Small stars mark fainter bands, which may correspond to silent segregating insertions (see text). Line marked as 33:2 serves as a positive control and contains DNA from the homonymous transformed line of *C. capitata* (Loukeris et al., 1995b).

Detailed Description of the Invention

Transformation efficiency, expressed as percentage of individuals giving transformed progeny, is a crucial parameter in designing strategies for transgenesis, especially for species that are difficult to breed. Mobile element mediated transgenesis is usually based on two components; a transposon and the homologous or cognate transposase. For mobile elements of the *Tc1/mariner* family, the presence of these two components during early embryogenesis is considered to be necessary and sufficient for integration of the transposon into host chromosomes, since transposases of *Tc1*, *Mos1* and *Himar1* can also catalyse transposition *in vitro* (12-14). However, there is evidence that transpositional activity may not be proportional to the amount of transposase present; high concentrations of transposase may inhibit transposition *in vitro* (33) and *in vivo* (15, our unpublished results). This work shows that the use of *in vitro* synthesised *Minos* transposase mRNA can result in high transformation efficiencies in both species that were tested, *D. melanogaster*, and the medfly *C. capitata*. Until now, two sources of *Minos* transposase have been employed for transformation of these species: A stable chromosomal integration of a transposase-expressing construct in *Drosophila*, (15) and transposase-expressing plasmids in *Drosophila* and *Ceratitis* (16).

A transformation frequency of 3.2% was accomplished in *Drosophila melanogaster* by injecting pMiw1, a non-autonomous *Minos* transposon marked with a wild-type version of the *white* gene, to pre-blastoderm embryos carrying a chromosomal source of transposase (15). Similar transformation frequencies (ca 1-6%) have been reported for *Minos*-mediated transformation of *Drosophila*, using the same transposon combined with a transposase expressing (helper) plasmid (15). The efficiency of transformation in these cases depends on a) the levels of transposase in germ line nuclei and b) the transformation procedure itself. Transposase levels may vary according to the promoter that drives its expression and, in the latter case, the amount of plasmid injected. Gradual improvements of technique have resulted in increased transformation efficiencies. In our hands, *Minos*-mediated transformation efficiency of up to 10% has been achieved in

Drosophila (unpublished data), using various transposons and the helper plasmid that was originally used by Loukeris et al. (15). The introduction of *Minos* transposase mRNA as a helper in *Drosophila* boosts transformation rates to 25%. This makes *Minos*-based transformation in *D. melanogaster* comparable in efficiency to P element mediated transformation.

Transformation rates of different insect species may vary widely, depending on the species and the transformation system. For example, transformation rates of 1% and 3-5% have been reported for Medfly with *Minos* and with *piggyBac*, respectively (16,9) of 4% and 8% for the mosquito *Aedes aegypti* with *mariner* and *Hermes* respectively (4,6), and 2% for the silkworm *Bombyx mori* with *piggyBac* (10).

Using *Minos* mRNA instead of a helper plasmid increased transformation rates of Medfly considerably. G0 Medflies are routinely tested in small groups under our laboratory conditions, because single pair mating of Medfly is not efficient, resulting in apparent low fertility. Consequently, our transformation rates represent minimum estimates. In this study, 14 of 20 cages, containing a total of 202 G0 male flies, yielded at least one transformed progeny. This gives a lower-estimate transformation rate of approximately 6.9% (14/202). The corresponding rates from cages containing G0 females is approximately 4.4% (8 of 9 cages, containing a total of 180 G0 females, see Table 5).

Integration rates, expressed as the frequency of appearance of different phenotypically detectable events, are an additional criterion of efficiency of transformation. Integration rates may actually be more informative than transformation rates, because they indicate the number of different independent lines that can be obtained from a given transformation experiment. Integration rates in the Medfly using *Minos* transposase mRNA are approximately 18% for G0 males and 13% for females. Southern analysis clearly indicated that the different eye phenotypes present among the G1 progeny from the same cage represent independent integration events. It is not possible to distinguish which of the independent phenotypes present in a cage represent a) multiple insertion events in the germ line of a single G0 or b) progeny from different G0's carrying single insertions, because the G0's were tested in groups. Southern analysis detected double insertions of the transposon in the progeny of 5 of the 12 G1's examined, suggesting that multiple integration events in the germ line of individual G0's are not rare (16,9). It is notable that these "second" insertions behave as "silent" events, i.e. there is no w+ phenotype associated with them. This is consistent with the observation that the white

gene is subject to strong position effects (34), which may result in total marker suppression (35). Our observation that out of 17 insertions only 12 were detectable though the eye phenotype, suggests that, in Medfly transformation with the MihsCcw transposon, up to 30% of the insertions may remain undetected. Consequently, transformation frequencies using the *white* marker are somewhat underestimated, and higher success rates can be expected with markers that may be less susceptible to position effects, such as the Green Fluorescent Protein (8).

A rapid assay was used to test activity of the *Minos* transposase mRNA in Medfly embryos. The assay is based on the observation that, as in other *Tc1/mariner* mobile elements, the parent chromatids are repaired after excision of *Minos* transposons, resulting in characteristic footprints at the site of the insertion; these events can be detected by PCR and characterised by sequencing (22). Excision/repair events can also be reproduced in plasmids that are introduced in insect cells and embryos expressing *Minos* transposase (17, 18, 19). We have shown that this assay can also be used for testing transposase activity in Medfly embryos, after co-injection with a donor plasmid and transposase mRNA. Such assays can be invaluable for assessing and optimising new sources of transposase or for testing established transposon systems in new host species, before embarking into time-consuming transformation experiments.

The stability of *Minos* transposon insertions in the genome in the absence of transposase suggests that there is no interaction between the *Minos* and other related mobile elements of the *Tc1/mariner* family that are present in the *D. melanogaster* and the Medfly genomes (15, our unpublished results). On the other hand, insertions can be mobilised upon expression of *Minos* transposase from a chromosomal position (16, 22). To determine whether mRNA-encoded *Minos* transposase expressed in early embryos can mobilise a *Minos* chromosomal insertion, a *Drosophila* transformant (C58) was used that carries a *Minos* integration in the X chromosome. This integration involves two copies of a *white*-marked transposon flanking a copy of the plasmid vector. Active *Minos* transposase induces mobilisation of either one or both transposons. Loss of both transposons in the germ line of C58 males was detected as exceptional daughters that had reverted to the *w* phenotype (15.9%). Transposition of the *Miw1* transposon from the X into an autosome was detected in the germ line of C58 males as non-white sons among their progeny (31.8%). Approximately 38% of male G0's, injected with 100ug/mL, gave at least one coloured-eyed female progeny with eye phenotype different than the heterozygous C58 phenotype. We consider that some of these females result from

excision of one, rather than both, of the two copies of the transposon in the C58 insertion. This could explain the observation that transposition events detected among G1 sons appear to be approximately twice as many as marker loss events detected among daughters of the same injected C58 males (31.8% vs 15.9%).

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Mobilisation of Minos transposons from chromosomal positions has been demonstrated before, using a genomic source of *Minos* transposase (22). These experiments showed that the frequencies of excision and transposition depend both on the helper chromosome and the configuration of the donor chromosome. The use of an RNA helper instead of a chromosomal source of transposase not only increases transposition frequencies (almost 3 fold in our system) but greatly simplifies the procedure of transposon remobilization since there is no need for elaborate mating schemes with strains expressing the appropriate levels of transposase. This would prove invaluable for mutagenesis screens, gene tagging and enhancer trapping both in *Drosophila* and in other insect species where the *Minos* system is active. Even in *Drosophila* where the *P*-element is routinely used for genetic analysis (23, 24, 28, 39) it is unlikely that a complete sampling of the genome will be achieved using only this element; *P*-element genomic insertion sites display a wide variation in their receptivity to insertion (40, 23, 41). Moreover, in non-*Drosophila* species of economic or of public health interest where *Minos* is active and not present in the genome, RNA-induced transposon mobilisation will allow genetic manipulations without the need to establish transgenic strains expressing transposase.

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25 Examples

Example 1: Introduction of *Minos* into the *D.melanogaster* genome. A *Minos*-based transposon in combination with a *Minos* transposase mRNA was introduced into preblastoderm *Drosophila* embryos by a microinjection procedure. Approximately 800 embryos were injected and the 154 adults (G0 generation) resulting from injected embryos were collected and backcrossed to *w* flies (Summarised in Table 1). Single male G0s were crossed to 4 *w* females whereas G0 females were crossed with 3 *w* males in groups of two except from 6 females that were used in single crosses. At least 6 of these G0 adults (2 males and at least 4 females) were sterile; the 38,452 progeny of the rest were screened for the presence of non-white phenotypes.

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A total of 691 flies with coloured eyes were recovered from 35 out of 114 different crosses. In many instances, w^+ progeny from the same cross, differed in eye colour. The eye colour varied from pale yellow to near wild type among different transformants presumably due to position effect depending on the insertion site of Miw1 transposon
5 (32). The same kind of variegation is typical with *P [mini-white]* as well as with *Minos [mini-white]* (15) insertions. This partial rescue makes the w marker very useful in detecting and sorting out different insertions. Based on these differences G1 transformants were classified into 60 distinct groups according to their eye phenotypes.

Analysis of the progeny of G0 flies (Table2) showed that although the efficiency of
10 transformation of the injected embryos is high (25% for the males and ~22% for the females) the actual frequency of G1 transformed progeny does not exceed 1.8%. Nineteen out of the 76 fertile G0 males gave at least one progeny with w^+ eye phenotype. At least 16 out of the 72 fertile G0 females gave w^+ G1 progeny but the number is probably higher because female G0s were backcrossed in groups of two. Eleven of the
15 single male G0s gave one event in their germ line, 5 gave two events and 3 gave three events. This results in an overall integration rate of ~39,5%. In G0 males, independent integration events in the germ line of each male G0 (Table 1) were screened from the eye phenotype and verified by Southern blots (data not shown). We cannot determine whether the different transformants from the same vial containing female G0's are
20 derived from a single or both G0 parents, and therefore an overall integration rate in G0 females cannot be determined from our data.

Example 2: Mobilisation of a *Minos* transposon using *Minos* transposase mRNA.

To determine whether *Minos* transposase expressed in early *Drosophila* embryos from
25 injected *Minos* mRNA can mobilise a *Minos* transposon from *Drosophila* chromosomes, we used an insertion of Miw1 on the X chromosome. This insertion consists of two Miw1 transposons arranged in tandem and separated by a full copy of the plasmid vector (15) (Fig. 1A) Two concentrations of mRNA (50 µg/mL and 100 µg/mL) were tested for their ability to induce a) excision of both transposons and b) re-insertion of the transposon in
30 an autosome. The *Minos* mRNA was introduced into *D. melanogaster* preblastoderm embryos by microinjections. Male G0 flies were backcrossed to w flies and G1 progeny was screened for exceptional eye phenotypes. Transposon loss in the germ line of the injected (G0) males was detected as female progeny that had reverted to the w phenotype. Transpositions to an autosome were detected as exceptional non-white eyed
35 sons (Fig. 1B). The non-white female progeny of the G0 males showed considerable variation in eye colour. For example, of the 44 fertile males injected with 100 µg/mL

mRNA, 17 (38.6%) gave at least one female progeny with either weaker or stronger eye colour than the typical heterozygous C58 phenotype. Weaker eye colour phenotypes presumably represent partial excision (i.e. excision of one of the two transposons present in C58); stronger phenotypes may represent transpositions to new sites.

5 Because of the difficulty to detect and analyse such complex events, only complete loss of the marker (white-eyed females) and transpositions to autosomes (non-white males) were scored in the progeny of injected males.

The results of this analysis are shown in Table 3. Approximately 16% of males injected

10 with 100 µg/mL mRNA had at least one excision event (w phenotype) among their female progeny. Excision frequencies dropped to 6% in males injected with 50ug/mL mRNA. Approximately 31.8% (14 out of 44) of males injected with 100 µg/mL mRNA, gave at least one exceptional w⁺ son among their progeny. The frequency of germ line transposition dropped to 6% in males injected with 50 µg/mL. Non-white eye phenotypes

15 of the exceptional males varied considerably, presumably due to strong position effects. In two cases, w⁺ sons of the same G0 male exhibited markedly different phenotypes, indicating independent transposition events. If these cases are considered, the overall frequency of transposition increases to 34% (for 100 µg/mL G0 males).

20 **Example 3: A rapid PCR assay for Minos transposase mRNA activity.**

A PCR-based assay was used to detect mRNA-encoded transposase activity in medfly embryos. The assay is based on the observation that after *Minos* excision the chromatid (or donor plasmid) can be re-ligated in the host cell (17, 22). Appropriate DNA primers were used (see Materials and Methods) which are expected to generate a 211 bp

25 fragment after precise excision of the transposon. As shown in Figure 2, under the PCR conditions that were used the full 2.2 Kb transposon sequence was recovered as well. Non-injected Medfly embryos did not give any of these bands. The full-length product was amplified from embryos that were injected with donor plasmids but no *Minos* transposase mRNA. The embryos that were injected with the mRNA/donor mix gave the

30 211 bp excision band along with the full length transposon sequence. These results were confirmed from one additional experiment with only donor injections and two additional experiments with mRNA/donor mix injections (data not shown). The results of the excision assay indicate that *Minos* transposase mRNA at a concentration of 100 micrograms per ml can be used as a source of active transposase in Medfly embryos.

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Example 4: Introduction of *Minos* into the *C. capitata* genome.

A *Minos*-based transposon (pMihsCcW) in combination with *Minos* transposase mRNA was microinjected into approximately 2400 medfly preblastoderm embryos. 382 adults (G0 generation) were collected and backcrossed to *w* Medflies. Groups of 10 G0 males were crossed to 20 *w* females whereas groups of 20 G0 females were crossed to 10 males (Summarised in Table 4). In total, 67,966 G1 flies were screened for the presence of a non-white phenotype (Table 5). A total of 665 flies with coloured eyes were recovered from 22 out of the 29 cages. The frequency of transformants among the G1 flies varied between 7.437% and 0.055%, with only 3 of the cages giving one transformed fly. Of the remaining 19 cages, 16 gave transformed progeny that varied in phenotype. Phenotypic variations included a) degree of coloration (from pale yellow to almost wild type) and b) colour variegation. Up to 8 different phenotypes were detectable in the G1 progeny of a single cage.

Genetic and molecular analysis was performed to determine the basis of the observed phenotypes. Twelve representative G1 male flies were backcrossed to *w* females and their progeny was scored for transmission of the eye colour phenotype and the presence of transposon sequences in their genome. Of these G1 flies analysed, 6 were from different G0 cages and 6 were from two cages that showed varying phenotypes. In all crosses, the eye phenotypes were transmitted as single dominant loci. No phenotypic variation was observed among the non-white progeny of each individual G1. Southern analysis of non-white G2 progeny from each cross was performed to define the molecular basis of these events. In this analysis each insertion event is expected to give two chimeric fragments, each consisting of one of the transposon ends plus genomic flanking sequences. As shown in Figure 3, seven of the examined G2's contain a single, distinct insertion of the MihsCcW transposon. In five cases pairs of (usually minor) bands were also detectable, indicating the presence of additional, segregating insertions. These insertions are presumably silent, because of the single-locus inheritance character of the *w*⁺ phenotype in the corresponding crosses. Southern analysis also showed that each of the two sibling G1 flies with different phenotypes that were analysed from G0 cage C44 (C44:1 and C44:3), and the four sibling G1 flies from cage C53 (C53:1, C53:2, C53:3, C53:4), contained distinct insertions. This result strongly suggests that the different eye phenotypes that were detected derive from independent insertion events.

Table 1. *Drosophila melanogaster* germline transformation using *Minos*-mRNA as a helper. An overview of the transformation experiment.

injected embryos	Hatched larvae	SEX	Adults (crosses)	Fertile G0's (crosses)	G1 Progeny	Non-white G1's	Varying non-white eye phenotypes
		males	78 (78)	76 (76)	26,981	483	30
~800	299	females	76 (41)	- (38)	11,471	208	30

Table 2. *Drosophila melanogaster* germline transformation using *Minos*-mRNA as a helper. Presentation of the 35 G0 crosses that had at least one transformant among their progeny.

G0	Percentage				G0				Percentage			
	MALES		of		FEMALES		of		Total		of	
Vial	Total	Non-white	G1's	non-white	Varying	Vial	Total	Non-white	G1's	Non-white	Varying	Varying
number	G1's	G1's	G1's	G1's	non-white eye phenotypes	number	G1's	G1's	G1's	G1's	non-white eye phenotypes	non-white eye phenotypes
D3M:4	208	90	43.269%	3	D1F:12	150	23	15.333%	2			
D3M:45	199	54	27.136%	2	D3F:14	204	26	12.745%	3			
D1M:2	387	79	20.413%	1	D3F:3	406	36	8.867%	3			
D3M:20	382	61	15.969%	3	D3F:6	249	22	8.835%	3			
D1M:33	276	43	15.580%	2	D1F:4	152	13	8.553%	1			
D1M:5	296	36	12.162%	3	D1F:16	200	13	6.500%	2			
D1M:21	382	30	7.853%	1	D3F:11	479	30	6.263%	3			
D1M:25	475	32	6.737%	1	D1F:15	144	7	4.861%	2			
D3M:36	312	13	4.167%	2	D1F:20	447	19	4.251%	2			
D3M:35	368	15	4.076%	2	D1F:13	176	7	3.977%	2			
D3M:30	349	13	3.725%	2	D3F:4	270	6	2.222%	2			
D3M:7	245	7	2.857%	1	D1F:1	108	1	0.926%	1			
D3M:6	207	2	0.966%	1	D3F:16*	196	1	0.510%	1			
D3M:3	217	2	0.922%	1	D3F:9	503	2	0.398%	1			
D1M:24	421	2	0.475%	1	D1F:14	279	1	0.358%	1			
D1M:3	292	1	0.342%	1	D3F:8	353	1	0.283%	1			
D1M:1	307	1	0.326%	1								
D3M:1	379	1	0.264%	1								
D3M:41	611	1	0.164%	1								

* D3F:16 has only one female G0

Table 3. Mobilization of a *Minos* chromosomal insertion by *Minos*- mRNA injected in preblastoderm C58 *Drosophila* embryos.

RNA (μ g/ml)	fertile G0 males	% of G0 males with white-eyed daughters	Total female G1's screened	% of G1 females with white eyes	% of G0 males with non-white eyed sons	Total male G1's screened	% of G1 males with non-white eyes
100	44	15.909	8,301	0.446	31.818	7,808	0.935
50	50	6.000	9,627	0.117	6.000	8,053	0.037
0	15	0	1,560	0	0	1,402	0

Table 4. *Ceratitis capitata* germline transformation using *Minos*-mRNA as a helper. An overview of the transformation experiment.

injected embryos	Hatched larvae	SEX	Adults (crosses)	G1 Progeny	Non-white G1s	Varying non-white eye phenotypes
		males	202 (20)	53,666	576	38
~2400	592	females	180 (9)	14,300	89	23

Table 5. *Ceratitis capitata* germline transformation using *Minos-mRNA* as a helper. Presentation of the 22 G0 crosses that gave at least one transformant among their progeny.

G0	Percentage of			Varying		Percentage		Varying	
MALES	Total	Non-white	Non-white	Non-white	Non-white	Total	Non-white	Non-white	Non-white
Cage	G1's	G1's	G1's	eye	eye	G1's	G1's	eye	eye
number				phenotypes	phenotypes	number		phenotypes	phenotypes
C39	3819	284	7.437%	2		C52	2017	50	2.479%
C53	1963	48	2.445%	5		C44	2019	25	1.196%
C45	261	6	2.299%	2		C55	2434	6	0.247%
C47	2112	41	1.941%	5		C60	1068	2	0.187%
C46	3248	45	1.385%	3		C42	1802	3	0.166%
C51	2244	29	1.292%	6		C62	957	1	0.104%
C48	3623	43	1.187%	4		C66	1190	1	0.084%
C38	4459	30	0.673%	1		C50	1822	1	0.055%
C58	3493	15	0.429%	2					
C43	3711	13	0.350%	2					
C64	2716	9	0.331%	2					
C40	2718	8	0.294%	1					
C59	2275	3	0.132%	1					
C41	2432	2	0.082%	2					

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CLAIMS

1. A method for genetically modifying a cell by transposon mobilisation, comprising the steps of:
 - 5 a) delivering to the cell a nucleic acid comprising a transposon of the Tc1/*mariner* class;
 - b) delivering to the cell a ribonucleic acid encoding a cognate transposase for said transposon; and
 - c) causing the ribonucleic acid to be translated to produce the cognate
- 10 transposase within the cell.
2. A method for mobilising a transposon resident in a host cell genome, comprising the steps of:
 - a) delivering to the cell a ribonucleic acid encoding a cognate transposase for
- 15 said transposon; and
- b) causing the ribonucleic acid to be translated to produce the cognate transposase within the cell, thus mobilising the resident transposon.
3. A method according to claim 1 or claim 2, wherein the transposon is selected
- 20 from the group consisting of Minos, mariner, Hermes and piggyBac.
4. A method according to claim 3, wherein the transposon is Minos.
5. A method according to any one of claims 2 to 4, wherein the transposon
- 25 comprises a nucleic acid sequence encoding a heterologous polypeptide.
6. A method according to claim 5, wherein the heterologous polypeptide is a selectable marker.

1/3

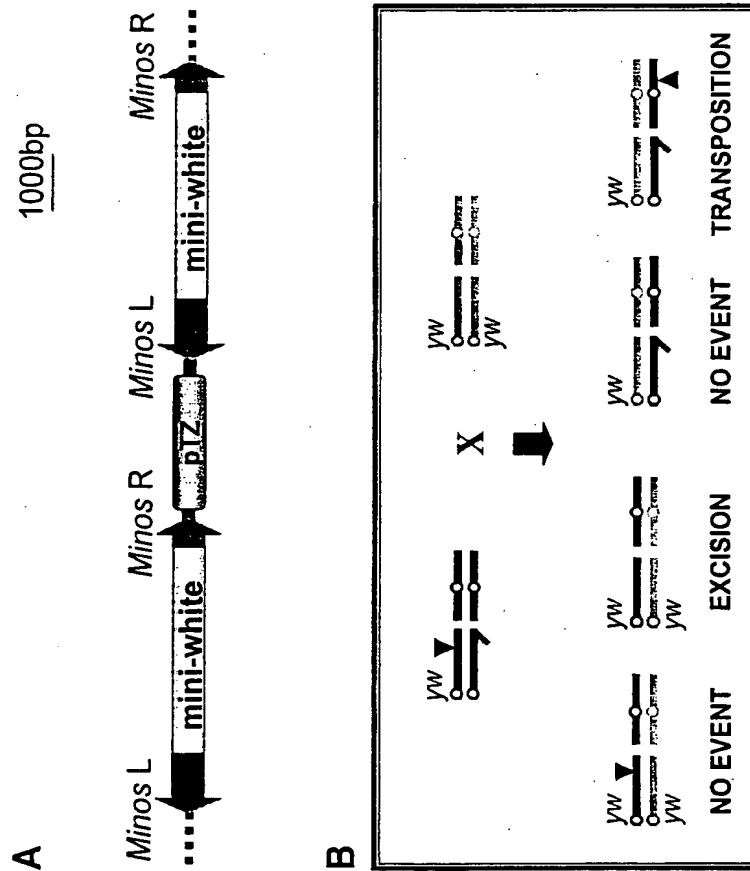


Figure 1

2/3

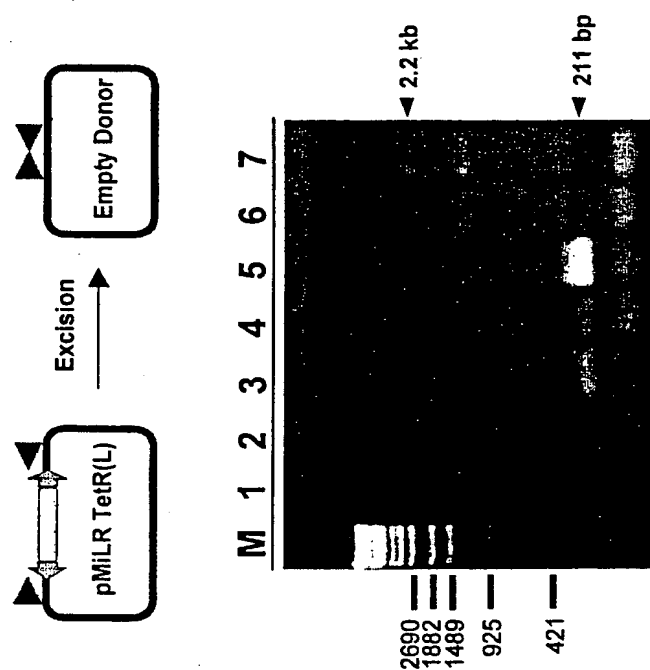


Figure 2

3/3

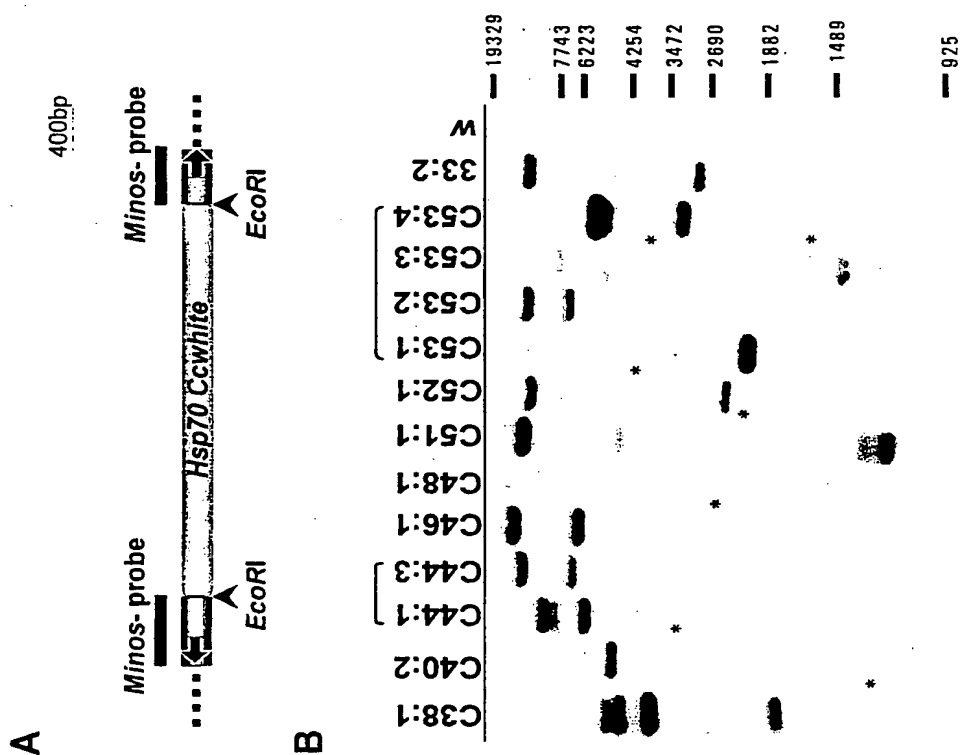


Figure 3

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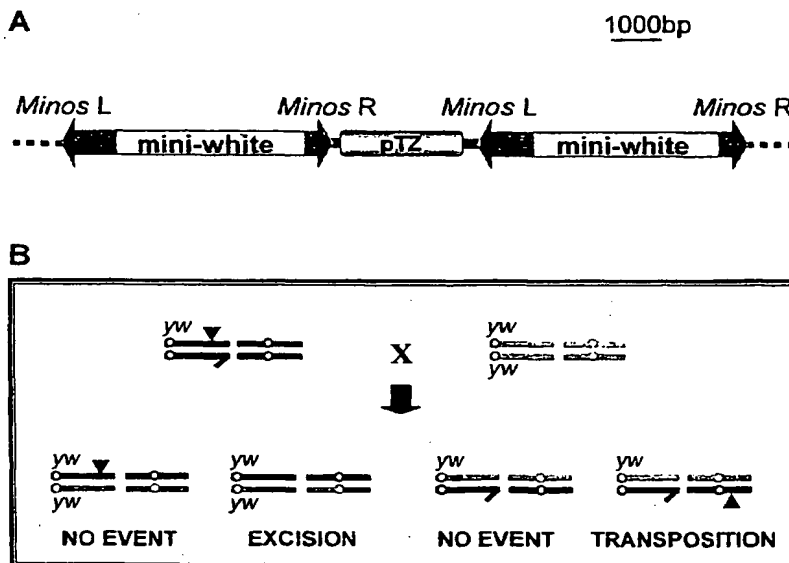
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(54) Title: METHOD FOR GENETIC MANIPULATION



(57) Abstract: Capped RNA corresponding to the spliced transcript of the *Minos* transposable element has been synthesised *in vitro* and shown to be active as a source of transposase for *Minos* transposon mobilisation. When co-injected into *Drosophila* and Medfly embryos, *Minos* mRNA can mobilise homologous transposons from plasmids into germline chromosomes. Injected mRNA is superior to injected plasmids expressing the transposase as a source of the enzyme, resulting in several-fold increases in transformation efficiencies. In a rapid test for mRNA activity, *Minos* mRNA has been shown to catalyse excision of a *Minos* transposon from plasmid DNA in Medfly embryos. High frequency mobilisation of a *Minos* transposon from the X chromosome into autosomes has also been demonstrated after injections of *Minos* transposase mRNA into pre-blastoderm *Drosophila* embryos. The rates of transposition (approximately 30 %) suggest that this is a powerful system for genetic manipulation.

INTERNATIONAL SEARCH REPORT

Internat. Application No

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A. CLASSIFICATION OF SUBJECT MATTER

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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	LOUKERIS T ET AL: "Introduction of the transposable element Minos into the germ line of Drosophila melanogaster" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 92, no. 21, 10 October 1995 (1995-10-10), pages 9485-9489, XP002164306 abstract ---	4
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